Gene-specific RT-PCR

This method was successful in our lab using prostate tissue and for our specific objectives. Investigators must be aware that they will need to tailor the following protocol for their own research objectives and tissue under study.

To generate first strand cDNA from mRNA, three priming methods are routinely used, depending on the quality of the starting RNA:

- Oligo (dT)-based priming is preferred when the RNA is of high quality, since first strand polymerization should be continuous from the 3' end of the transcript to the desired sequence regardless of the length of the 3' UTR.
- Random hexamer priming is recommended for fragmented mRNA (lengths <500 bases) where there may be discontinuity between the desired sequence and the poly A tail.
- A primer that is specific to the gene of interest is best when amplifying low abundance transcripts.

The NCI Prostate Group has found that the most efficient method for first strand cDNA synthesis of mRNA from microdissected samples, which may contain fragmented mRNA, uses random hexamers, as they circumvent the possibility of disruptions in oligo (dT)-based first strand cDNA polymerization.

A: Reverse Transcription

1. Reagents

1. DEPC-dH_2O (Research Genetics)
2. RNase inhibitor, 20 U/µl (Perkin Elmer)
3. dNTP, 250 µM (GenHunter)
4. First strand buffer (Life Technologies)
5. Random hexamer primers, 50 mM (Perkin Elmer)
6. Superscript II (SSII) reverse transcriptase (Life Technologies)

2. Method

The protocol below may be used for amplifying individual transcripts from RNA recovered from microdissected cell populations as described in RNA-based Studies of Microdissected Tissues.

> **TIP:** The number of cells needed to perform gene-specific RT-PCR from microdissected samples is highly dependent on the quality of the tissue sample and the abundance level of the transcript(s) of interest. One thousand cells is recommended as a good starting point for gene-specific RT-PCR studies. However, reliable amplification can frequently be achieved using substantially fewer cells.

1. To RNA pellet, add 10 µl DEPC-H_2O and 1 µl RNase inhibitor.

2. Resuspend the RNA pellet with gentle tapping.

3. Quick spin.

4. Aliquot 5 µl into 2 sterile tubes for (+) and (-) RT reactions.

5. For each batch of samples, prepare additional control tubes as follows, using either high-quality RNA or DEPC-dH_2O in place of the 5 µl sample RNA:

Control Type	(+) RT	(-) RT
Positive	High-quality RNA	High-quality RNA
Negative	DEPC-dH_2O	DEPC-dH_2O

6. Prepare sufficient volume of the following +/-RT master reaction mixtures for all reaction tubes.

(+) RT master reaction mixture/tube	
1.0 µl	DEPC-dH_2O
2.0 µl	First strand RT buffer
1.0 µl	dNTP
0.5 µl	Random hexamer primers
	Total volume = 4.5 µl

(-) RT master reaction mixture/tube	
1.5 µl	DEPC-dH_2O
2.0 µl	First strand RT buffer
1.0 µl	dNTP
0.5 µl	Random hexamer primers
	Total volume = 5.0 µl

8. Aliquot either 4.5 µl or 5.0 µl of the relevant master mix to the (+) and (-) RT tubes.

9. Incubate at 65°C for 5 minutes, then at 25°C for 10 minutes.

10. Add 0.5 µl SSII to all (+) RT tubes only.

11. Incubate all tubes at 25°C for 10 minutes, then at 37°C for 40 minutes.

12. Incubate at 95°C for 5 minutes to denature the SSII.

13. Quick spin.

B: Polymerase Chain Reaction (PCR)

1. Reagents

1. Advantage 2 Polymerase Mix, containing Taq polymerase and PCR buffer (Clontech)
2. dNTP, 25 µM (GenHunter)
3. Forward primer, 10 µM
4. Reverse primer, 10 µM
5. dCTP α-33P (or α-32P) (10 mCi/ml)
6. DEPC-dH_20 (Research Genetics)
7. High density TBE sample buffer, 5X (Novex)

2. Method

TIP: As with any PCR reaction, it is good practice to run duplicate or triplicate samples to ensure the validity of the PCR result.
TIP: Components and cycling will depend on individual template and primers.

1. Aliquot 3 µl of each cDNA sample into a sterile PCR tube.
2. Prepare sufficient volume of PCR master reaction mixture for all reaction tubes and add 7 µl to each tube.

PCR master reaction mixture/tube	
1.0 µl	PCR Buffer
0.8 µl	dNTP
0.2 µl	Forward primer
0.2 µl	Reverse primer
0.2 µl	dCTP α-33P (or α-32P) see **TIP below**
0.2 µl	Taq DNA polymerase, 5 U/µl
4.4 µl	DEPC-dH_20
	Total volume = 7 µl

3. **TIP:** The PCR protocol presented here includes incorporation of radioactivity into the PCR products. Radioactivity is necessary for visualization on a denaturing acrylamide gel for low abundant transcripts or when PCR product patterns are complicated (i.e., polymorphic markers for LOH). The amount of radioactivity used in the above protocol often results in visible products in less than 2 hours

exposure. For abundant mRNAs, it may be possible to discern products on an ethidium bromide treated agarose gel (replace ^{32}P or ^{33}P volume with water).

4. PCR Cycling Conditions:

Cycles	Temp. (°C)	Time
1	95	2 min
35	95 X* 72	15 seconds 45 seconds 5 min
1	72	10

5. X* is annealing temperature, dependent on the primer used.
6. Store the PCR products at 4°C or continue to step 5.
7. Pour a 6% polyacrylamide sequencing gel while the PCR is cycling.
8. After cycling is complete, add 2.5 µl sample buffer (5X) to samples
9. Denature samples at 95°C for 3 minutes and place directly on ice.
10. Load 3.5 µl sample on gel and run at 1600 V to desired distance.
11. Dry gel and expose to phosphoroimager screen or film as described under LOH protocol.